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Gingival Retraction Procedure is Associated with Increased Gene Expression of TNF-α and TNF-α Receptors: A Pilot Study

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Authors' contributions

This work was carried out in collaboration of the authors. The experimental design, protocol and writing of the manuscript were carried out by authors SW and AS. Authors GH and ZH designed and performed the TNF-a analyses. All authors read and approved the final manuscript.

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ABSTRACT

Background: Gingival retraction procedure (GRP), a regularly practiced clinical procedure, displaces the gingiva from around the tooth, temporarily. It has been reported that increased tumor necrosis factor-alpha (TNF-α) levels in gingival crevicular fluid (GCF) were observed even after 30 days following GRP. TNF-α is a pro-inflammatory cytokine found in periodontally-diseased tissues and is associated with loss of connective tissue and bone. The effects of (TNF-α) are tightly regulated by TNF receptor-1 (TNFR1) and 2 (TNFR2). The object of these experiments was to test the hypothesis that up-regulation of TNF-α expression is associated with increased gene expression of its receptors, (i.e., TNFR1 and TNFR2).

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Methods: In a GRP rat model, the gingival Index (GI) was recorded, the expressions of TNF-α, and associated genes TNFR1, and TNFR2 in gingival tissue were measured and hematoxylin and eosin (H&E) histological examination was performed.
Results: Expression of TNF-α and TNFR2-related genes demonstrated similar profiles. Both paralleled the profile of the GI. They peaked at day 1 after GRP, and were reduced but still remained at a higher level as compared to sham control at day 3 after GRP, and then returned close to normal at day 7. On the other hand, TNFR1 gene expression peaked at day 3 and remained elevated at day 7 after GRP.
Conclusions: The results indicate gene expressions of TNF-α and TNHR2 are closely associated with the time course of the gingival injury from the retraction procedure, while TNFR1 gene expression may have further long-term effects.

Keywords: TNF-α; gingival retraction; TNHR1; TNHR2; inflammation.

1. INTRODUCTION

Gingival retraction is a routine clinical procedure performed during prosthodontic treatment to insure an accurate impression of the marginal areas of the abutment teeth. A cotton cord is packed temporarily into the gingival sulcus to dilate the marginal gingiva and separate it for the impression material. Although the epithelial attachment and gingival fibers are separated from the tooth surface due to the stress caused by retraction cord, this injury is reversible [1]. However, Feng et al., have shown that increased concentration of tumor necrosis factor alpha (TNF-α) in gingival crevicular fluid (GCF) was associated with gingival retraction by cord, accompanied by an increased gingival index (GI) score [2]. Enhanced inflammatory mediators in the gingival tissues may be ascribed to the injury of gingival tissues that occurred during the retraction procedure [3]. TNF-α is a cell signaling protein (i.e., cytokine) involved in inflammation, including the gingival inflammation associated with gingivitis and periodontitis [4]. Feng et al, reported that the increased TNF- α concentration in GCF reached its highest level ~24 hours after GRP, and remained elevated for 3 days after GRP. Likewise, the scores of the gingival index were elevated, reflecting the nature of the injury associated with GRP that subsequently healed clinically in 2 weeks [2].

TNF-α exerts its pro-inflammatory and cellular homeostasis functions by binding two different receptors: (i) TNF-receptor 1(TNFR1; also known as TNFR55) and (ii) TNF-receptor 2 (TNFR2; also known as TNFR75) [5]. Both TNFR1 and TNFR2 are associated with the cell membrane and are present in soluble forms. Soluble TNF-α receptors, sTNFR1 and sTNFR2, are proteolytically shed upon inflammatory stimuli and modulate TNF-a-related activity [6]. It has been reported that TNF-α up-regulates the gene expression of TNFR2 in human gingival fibroblasts, but does not affect TNFR1 [7].

To further extend the observations of Feng et al., the purpose of this study was to investigate, in a rat animal model, the gene expression of TNF- α, TNFR1 and TNFR2 in gingival tissue after GRP accompanied with GI to find the relationship between gingival injury induced by GRP and pro-inflammatory cytokine TNF- α and its receptors. It is postulated that the interactions between TNF-α and its receptors (i.e., TNFR1 and TNFR2) can potentially regulate inflammation and tissue destruction in gingival tissues after GRP.

2. MATERIALS AND METHODS

Animals: Thirty Sprague-Dawley male rats (250-300 g body weight) were used. Rats were anesthetized with ketamine (50 mg/kg/xylazine solution (5 mg/kg, i. p), and a fine cord approximately 0.5 mm in diameter was pushed into the space between the gingiva and the tooth to a depth of 1 mm for the two central incisors of the lower or upper jaw using a fine bladed, blunt instrument. It was left in place for 15 minutes. Before the animal awoke, the cords were removed. This procedure is similar with what is done in a human patient where the cord is kept in place for 15-20 minutes. In order to define the time course of gene expression of TNF-α and its receptors in the gingival tissue after cord pack, 4 different time points, a sham control, 1 day, 3 days and 7 days after GRP were selected. The sham control group was used as a baseline inflammatory marker. These animals received anesthesia and cords were placed, but without packing. At each time point, the GI was recorded, and gingival samples were obtained. Sham control rats were sacrificed after the sham

procedure. Collected samples were kept in -80°C freezer for RNA extraction.

Gingival Index (GI): The GI used in this experiment was modified from the gingival index described by Loe [8] using criteria of gingival color, tissue edema and structure (Table 1).

Histologic Analysis of Gingival Tissue: After harvesting, gingival samples were immediately fixed in 10% neutral buffered formalin for greater than 48 hours. Histological slides were prepared by histological laboratory. Briefly, samples were decalcified in EDTA-glycerin-PBS (pH 7.0). Then they were washed with PBS, underwent a paraffin wax procedure and the tissue blocks cut into 6 micron sections. After deparaffinization and rehydration, tissue sections were stained with hematoxylin and eosin. Tissue slides were imaged digitally and analyzed.

Primer Design: The primer design was modified from our previous publication [9,10]. Briefly, mRNA sequences of TNF-a, TNFR1, TNFR2 and control factor hypoxanthine-guanine phosphoribosyltransferase (HTPA) were selected from GenBank NCBI. Every applicant was crossing an intron. Primer melting temperatures (T_m's) for all selected primers ranged from 54°C to 56°C. The lengths of the amplicons ranged from 99 to 101 bases pairs. These primers were designed by a custom-made computer program to exclude interactions between primers and allow the panel of primers to amplify in an RT-PCR reaction tube. Primer sequences are list in Table 2.

RNA Extraction: Rat gingival tissues of about 50 mg were suspended into 100 µl extraction solution (2 µl RNasin Ribonuclease inhibitor, 20 µl of 5× QIAGEN OneStep RT-PCR buffer, 78 µl H_2O). Tissue was grinded with a pellet pestal in a microcentrifuge tube manually for 1 minute and followed with three repeating cycles of alternating 30 seconds incubation between -80°C and 37°C water baths. Then, samples were kept in -80°C freezer until used for One-Step RT-PCR.

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One-Step RT-PCR: One-step RT-PCR was modified from published methods [9,10]. It was carried out in a 50 µl reaction containing 10 µl of 5× QIAGEN OneStep RT-PCR buffer, 10 µl of 5× QIAGEN Q-Solution, primers (1 µM each) for all the 4 mRNA species, the four dNTPs (400 µM each), 60 units/1.5 µl of RNasin Ribonuclease Inhibitor (Promega) and 2 µl QIAGEN OneStep RT-PCR Enzyme Mix. Samples thawed on ice for 10 min. One µl supernatant was pipetted and added into the reaction mix to achieve the 50 µl final volume. The reaction started at 50°C for 40 min to synthesize the selected cDNAs, and then were heated to 95°C for 15 min to inactivate the reverse transcriptase and activate the *Taq* DNA polymerase followed by 15 PCR cycles.

Table 1. Criteria for the gingival index system

Gingival Index
0 = normal gingival.
1 = mild inflammation: slightly change in color and edema; no bleeding on probing.
2 = moderate inflammation: redness, edema, and glazing; bleeding on probing.
3 = severe inflammation: marked redness and edema; ulceration; tendency to spontaneous bleeding.

Table 2. Primers used in the experiment

Transcript Name	**Direction**	**Primer Sequence (5'->3')**	**Product Size (bp)**	**Accession**
Brie	Forward	ACACTCAGATCATCTTCTCA	100	X66539
	Reverse	CACGCTGGCTCAGCCACTCC		
TNFR1	Forward	TTCGCAGAACCACGTCAGAC	100	NM_013091
	Reverse	CACGGTGTCCATGTCAGCTT		
TNFR2	Forward	CGTCGAACTGCAGCTGTGG	99	AY191269
	Reverse	TGTGAGATCTGGCACTGGTT		
HPRTA	Forward	GGTCCATTCCTATGACTGTA	101	M86443
	Reverse	AGTTGAGAGATCATCTCCAC		

Quantitative Real Time-PCR: Quantitative RT-PCR (qRT-PCR) was performed using the SYBR Green PCR assay system (Applied Biosystems). A 10 µl final reaction volume was achieved by 5 µl 2x SYBR Green PCR Master Mix, 1 µl PCR primer pair, 1 µl RT-PCR product and 3 µl H_2O. PCR amplifications were performed on an LightCycler 96 system (Roche). Relative gene expressions were calculated by using the $2^{-\Delta\Delta Ct}$ method [11]. The HTPA gene was used as an internal endogenous control. The qRT-PCR assay for part of the samples were performed by a 7500 Real Time PCR System (Applied Biosystems). Before integrating data obtained from two different qRT-PCR machines, a mathematic normalization was applied on these data.

Statistics Analysis: The qRT- PCR results were tested with a One-way Analysis of Variance (ANOVA) to compare the mean differences among the four time points. A Tukey HSD Post Hoc was performed when the One-way ANOVA was statistically significant. A non-parametric test, Kruskal-Wallis, was used to analyze the Gingival Index data. A Bonferroni correction was applied for the pairwise comparisons. The significance level was set at $p < 0.05$ for all tests.

3. RESULTS

Rat GI: Pathological changes occurred in the gingival tissues on day 1 after GRP, which included redness, edema, and light bleeding (Fig. 1). The GI score increased and reached its peak on day 1 after GRP during the 7-day experimental period (Table 3). On day 3 after GRP, GI decreased 50% compared to that of day 1. The gingival tissues recovered their normal appearance on day 7 after GRP.

Table 3. Gingival index

	Sham	Day 1	Day 3	Day 7
Medium	0	2	1	0
Range	0	2-3	0-2	0
Sample size	5	5	6	5

TNF-α expression: The gene expression of TNF-α in the gingival tissues increased on day 1 after GPR and was partially decreased on day 3 (Fig. 2A). On day 7, the TNF-α level was close to pretreatment levels.

TNFR1 and 2 gene expression: The gene expression of TNFR1 did not follow the profile of GI and TNF-α. It's peak was on day 3 (Fig. 2B). It displayed a higher level than that of sham control at the day 7 after GPR. The gene expression profile of TNFR2 appeared to be similar with that of TNF- α and the profile of GI (Fig. 2C and 2A; Fig. 1).

Histological Analysis: Analysis of the histological sections revealed that on day 1 after GPR, polymorphonuclear neutrophils (PMN) numbers were remarkably increased in the gingival tissues (Fig. 3B). On day 3 after GPR, the PMN population still appeared to be higher than that of sham control (Fig. 3C, 3A, and 3D). On day 7, the intensity of the inflammatory intensity in gingival tissue was reduced to the sham control level (Fig. 3D and 3A).

4. DISCUSSION

TNF-α is a pro-inflammatory cytokine presents in gingival tissues. It is regulated by binding two different receptors. Enhanced gene expressions of TNF-α and its receptors (i.e., TNFR1 and 2) indicates increased tissue inflammation. TNF receptors are expressed in a spectrum of cell types including macrophages, PMNs, lymphocytes and fibroblasts [12]. Both receptors have a role in the regulation of TNF-α in gingival tissues. In the present study, the TNF-α gene expression displayed a similar profile as the GI. Both the clinical markers and the TNF-_α gene expression peaked day 1 after GRP and were decreasing on day 3 (Fig. 1; Fig. 2A). Feng et al., reported a similar significant increase in GI and TNF-α in GCF after cord packing which began to decrease day 3 after GRF [2].

Histological analyses of rat gingival tissue in the affected sites revealed an intense PMN infiltration in connective tissue on day 1 after GRP (Fig. 3B). Increased numbers of PMNs in gingival tissue correlates clinically with an increase in the GI score (in terms of redness and edema) and also with increases in TNF-α and TNFR gene expression [13]. Although increased number of lymphocytes was still present in the gingival tissues, this was decreased by day 3 after GRP (Fig. 3C).

The gene expressions of TNFR1s and TNFR2s were significantly increased. The increase in receptor TNFR2 paralleled the increase in TNF-α gene expression and GI and as well, returned to baseline level by day 7. However, the titer of TNFR1 was maximally elevated on day 3, and did not return to baseline level by day 7. The explanation for these observations may be

related to the functioning of the TNF-α system. When an acute tissue injury occurs, TNF-α is initially activated through the complement system that increases the permeability of the endothelium and chemotactically results in adhesion of inflammatory cells to the sides of the vessels and their subsequent migration to the site of injury [14]. In the case of cord packing into the marginal gingiva, Sharpey's fibers are separated, the epithelium is stripped from the inner wall of the gingival margin and the gingival connective tissue exposed [3]. The initial acute response is mediated by PMN inflammatory cells. Histologically by day 3, the PMN infiltrate was reduced in intensity, but a number of lymphocytes were still observed. While TNFR1 receptor is found on many cell types, TNFR2 receptor is observed mainly on lymphocytes, monocytes, and endothelial cells [15].

TNFR2 is an inducible factor and Its gene expression can be induced by TNF-α and IL1- β. On the other hand, TNFR1 gene expression cannot not be induced by TNF-α and IL1- β [16]. TNFR2 may regulate the activation of TNF- α by its soluble form, sTNFR2. Subsequent to an acute injury, TNF-α is released. As the concentration of TNF-α increases, the extracellular portion of TNFR2 separates from the cell membrane, now termed sTNFR2, and combines with the TNF-α molecule. The sTNFR2 binds to TNF-α and competes with cell membrane-bound TNFR2, connecting to TNF-α, thereby blocking the TNF-α effects on the target cells [12]. The competition of sTNFR2 with cell membrane-associated TNFR2 indicates a probability that when TNF-α attacks target cells and there is enhanced gene expression of TNFR2, the increased sTNFR2 in turn blocked TNF-α activity [15]. Consequently, a reduction of inflammatory markers began when TNFR2 gene expression reaches its peak on day 1 after GRP (Fig. 2A, 2B). The time course of gene expression of TNFR2 followed a similar pattern as that of the TNF-α ligand and the GI.

A possible explanation for these observations lies in the structural and functional characteristics of TNFR1 which may have important clinical implications. TNFR1, attached to the cell membrane, in its intracellular moiety contains a "Death Domain", a molecular sequence that activates pathways that enhance upregulating proliferation, chemotaxis and adherence of PMN's, macrophages and lymphocytes [15]. In regions of inflammation the results are apoptosis and necroptosis of both invading bacteria and gingival structures as collagen, fibroblasts and osteocytes. Through a pathway that recruits a series of proteins including kinases that allows ligand transfer of the intracellular portion of the TNFR2 to the TNFR1 string, gene expression and intracellular production of TNF-α occurs that allow for increased tissue destruction [16]. Both the initiation of this process and its continuation past the acute phase of the injury were in the findings of Feng where continued upregulation of TNF-α was reported [2].

The results of this process are loss of connective tissue and bone [17]. Soluble forms of these receptors do not participate in these processes as they are found in the extracellular compartment and have little effects intracellularly upon gene activation. In fact, it seems that soluble forms of both receptors serve to downregulate TNF-a and reduces the its effects by joining together with TNF-α and prevent activation of the pathways previously described. TNFR2, it appears, can have a protective function particularly if the TNF-α titer is low. Under these circumstances it serves, through RANKL, to destroy invading bacteria. It is only when the TNF-α titer increases and most of the available TNFR receptors are bound to the cell membrane that destruction of connective tissue and bone occur [17].

It is interesting that a gene mutation of TNFR2 (1587G) polymorphic allele is associated with severe chronic periodontitis in Japanese [18]. Graves et al. reported that local injection with sTNFR2 could inhibit progression of inflammatory cell infiltration toward alveolar bone in a Macaca fascicularis primate model of experimental periodontitis [19]. The same research group further reported that sTNFR2 could significantly reduce loss of gingival connective tissue attachment and alveolar bone heights in the same model of experimental periodontitis [20]. It would also explain the observation that although the mean TNF-α gene expression for the clinical cohort returned to baseline level, for a number of the subjects individually, the elevated levels of TNF-α did not return to baseline even by the end of the 30 day experimental period. Residual activity of the TNF-α system was still occurring, although at a lesser level.

While this mechanism is partially speculative, it is clear from these studies, that the gingival retraction procedure should be approached with respect. It must be recognized that cord

retraction produces an acute injury that may or may not be entirely reversible [21]. Clinicians should approach gingival retraction with caution particularly in cases where there is a thin biotype and the vascular supply is limited. The subjects selected in the human study by Feng et al. presented with healthy gingiva and a hemostatic agent was not employed. Clinically, patients with poor biofilm control or active gingival or periodontal inflammation as well as those who received an application of astringents during the retraction procedure present further risk factors that may result in extensive tissue damage and extended release of TNF-α [22]. Multiple packings of cord to re-impress areas that were not adequately recorded should be avoided whenever possible for a similar rationale.

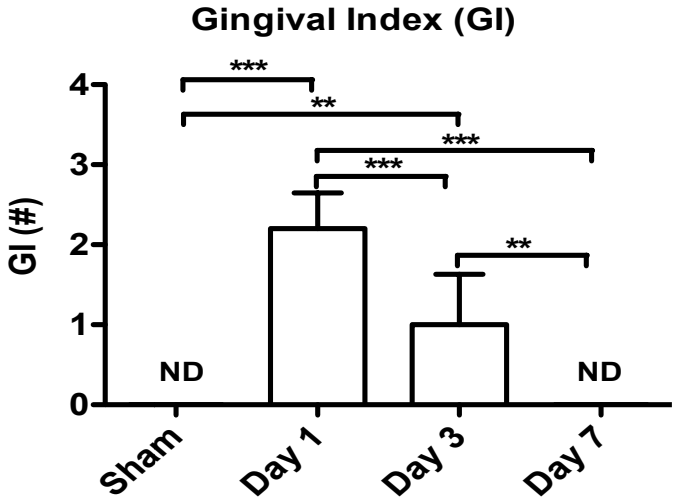

Fig. 1. The time course of increases in the gingival index resulting from a 15 minute cord placement. The gingival index was markedly increased on Day 1. This response gradually decreased and returned to baseline by Day 7. ** indicates ($p < 0.01$), * indicates ($p < 0.001$)**

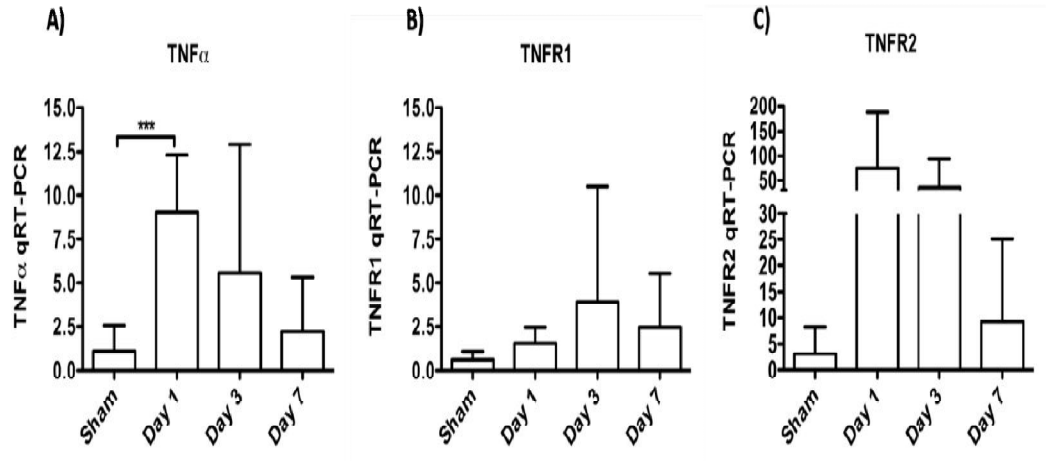

Fig. 2. Gene expression of (A) TNF-α, (B)TNFR1 and (C) TNFR2 assayed by qRT-PCR. * indicates ($p < 0.001$), Bar represents the mean of qRT-PCR reading of samples from each rat of the group**

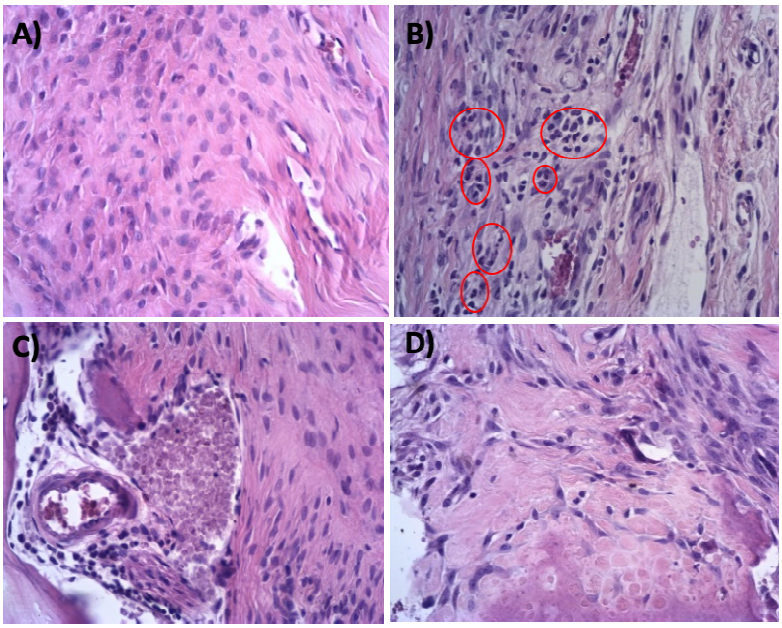

Fig. 3. Histological images indicating inflammatory response in gingiva to cord placement. A) Sham control. Fibroblasts predominate the gingival connective tissue. A few monocytes are observed. B) 24 hours after GRP. Increased infiltration of PMNs in gingival connective tissue. C) 3 days after GPR. PMN and monocytes infiltration are observed in connective tissue. D) 7 days after GPR. PMNs are scarcely observed in gingival connective tissue

5. CONCLUSION

In these experiments we observed that enhanced TNF-α and TNFRs is associated not only with bacteria-dependent periodontitis but also with the physical injury associated with gingival retraction. These experiments further revealed the dynamic time courses of gene expression of TNF-α TNFR1, and TNFR2 in a GRP rat model. Based on the gene expression profiles, it is clear that TNF-α is an early expressed factor after GRP, indicating that it could be a target to limit the effects of the damage from the procedure pharmaceutically. An understanding of the mechanisms underlying physical injury of gingival retraction may provide a guide for clinical management. However, further research is required to understand the roles of cytokines and prostaglandin derivatives associated with tissue retraction and gingival inflammation and recession.

CONSENT

It is not applicable.

ETHICAL APPROVAL

These animal experiments were reviewed and approved by the IACUC (Institutional Animal Care and Utilization Committee), Newark campus, of Rutgers Health Sciences.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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